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Improvement of Antioxidant and Immune Status of Atherosclerotic Rats Adrenaline and Egg-Yolks -Induced using Cardamom-Rhizome-Ethanolic-Extract: An Initial Study of Functional Food

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Abstract

The aimed to improve the activity of SOD and reduce the levels of MDA, CRP, and IL-6 of atherosclerotic-rats using cardamom-rhizome ethanolic-extract (CREE). A total of 28 *Sprague-Dawley* rats, aged 2-3 months, 180-250 g were injected with adrenaline, and given the egg-yolks for 3 weeks. The rats were divided into 4 groups, 7 rats each, I, were given CREE; II, statin; III, CREE+statin; and IV, feed-rats, 2 weeks. Blood samples were taken at 0, 1 and 2 weeks. CREE significantly increased of SOD, and decreased levels of MDA, CRP and IL-6. Future, the cardamom rhizomes would be formulated of functional-drinks.

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Keywords: cardamom-rhizome; SOD-MDA; CRP-IL-6; atherosclerotic-rats; functional-drinks

1. Introduction

There are many factors lead to atherosclerosis, such as oxidative stress (Wellen and Hotamisligil, 2005) and inflammation (Larsen et al., 2007; Winarsi and Purwanto, 2010). Oxidative stress related to the number of free radicals in the body that suppress antioxidant and immune status, as well as making the patient's condition worse. Oxidative stress is described by high levels of malondialdehyde (MDA) and low activity of Superoxide Dismutase (SOD), catalase, and Glutathion Peroxidase (GSH-PX) (Winarsi et al., 2012; Winarsi et al., 2013). In clinical

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studies, markers of inflammatory showed high levels of hs-CRP and IL-6 reflects the poor immune status. That markers contribute to the early stages of coronary artery disease (Lind, 2003; Luc et al., 2003). CRP is hepatic inflammatory product that works regulated by cytokines IL-6. IL-6 is a proinflammatory cytokine messenger secreted by macrophages and smooth muscle cells in atherosclerotic lesions. It was also reported that CRP is the most important marker (Danesh et al., 2004), as well as IL-6 since they are consistent predictors in cardiovascular events, such as myocardial infarction, stroke, and peripheral arterial disease in the future. Clinical studies show that the level of inflammation plays an important role in the pathology of atherosclerosis. Because of the inflammatory marker, high or low immune status associated with high risk of atherosclerosis.

Usually, atherosclerosis and symptoms resolved with medication. Atherosclerosis is a chronic condition, and then the long-term using drugs often have a negative impact. Its need to find alternative ways of natural ingredients rich in antioxidants, that can inhibit the development of atherosclerosis, such as cardamom rhizomes. Many people only know cardamom because the seed has a high economic value. All parts of this plant is often used as traditional medicine, by the people in a particular area.

Winarsi et al. (2012) has been extracting cardamom leave, it contains flavonoids of 129.6 mg / g and Vitamin C 19.22 mg / g. Leave of cardamom is able to lower the atherogenic index (Winarsi et al., 2013a), antidiabetic, helps weight lost and hypocholesterolemic to alloxan-induced *Sprague Dawley* diabetic rats (Winarsi et al., 2014). In a preliminary study, IC_{50} value of cardamom rhizome is the lowest compared to cardamom leaves and stems (Winarsi et al., 2012). According to Brand-Williams et al. (1995) IC_{50} is the concentration of antioxidant compounds that cause 50% of radical loses its radical character. Based on the IC_{50}, it appears that the antioxidant potential of rhizomes are greater than most other parts of the cardamom plant. Winarsi et al. (2015) reported that the flavonoid of CREE is nearly 3-fold of Cardamom leave ethanol extract (CLEE). Some researchers claim that the antioxidant flavonoid compounds capable of suppressing inflammation and improve immunity (Winarsi et al., 2013b; Zhang et al., 2011), protecting the endothelial cell membrane by means of modulating the lipid profile (Winarsi et al., 2013a), vasodilatative, and prevents the development of atherosclerosis (Clarkson et al., 2001). This study aimed to reveal the antioxidant potential of CREE on SOD activity, and MDA, CRP and IL-6 plasma levels to adrenaline and egg-yolks -induced atherosclerosis rats.

2. Materials and methods

2.1. Preparation of Cardamom Rhizomes Ethanolic Extract (Winarsi et al., 2012 modified).

Cardamom rhizome was washed, thinly sliced, dried and then ground into flour rhizomes cardamom (FRC). FRC maceration performed using 96% ethanol overnight, then the next morning was filtered with Whatman 41 paper soaked in ethanol precipitate, and then filtered. Such treatment was done repeatedly until a clear filtrate was obtained. By using a rotary evaporator, the ethanol in the filtrate was evaporated, then concentrated, called a Cardamom Rhizome Ethanolic Extract (CREE).

2.2. Preparation of Atherosclerosis Rats

The experimental animals were 28 female rats of *Sprague Dawley* (SD) strain, aged of 2-3 months, and weighed of 180-250 g. Rats were acclimatized for 7 days with rats fed and water *ad libitum*. To get the atherosclerotic rats, then the animal was injected with adrenaline (0.006 mg / 200 g BW) (Fadhilah and Prasetyo, 2001). To accelerate atherosclerosis, then after the adrenaline was injected, the rats were given a diet egg-yolks as much as 5 g / 200 g BW per day (Prasad et al., 2003) for 3 weeks. Otherwise suffered atherosclerotic rats when the levels of total-cholesterol were > 45.5 mg/dl and HDL-cholesterol were <35 mg/dl (Winarsi et al., 2013a).

2.3. Grouping Atherosclerosis Rats and Intervention

Atherosclerosis rats were randomly divided into 4 groups, 7 rats each. The four groups were treated as 1 = Atherosclerosis Rats were given feed Rats + CREE; 2 = Atherosclerosis Rats were given feed Rats + simvastatin

(statin); 3 = Atherosclerosis Rats were given feed Rats + CREE + statin; and 4 = Atherosclerosis Rats were given feed Rats only, with Intervention for 2 weeks.

2.4. Blood sampling and testing

Blood sampling was done three times, namely: baseline, one and two weeks after the intervention. One ml of blood was taken through *Plexus retroorbitalis* of the eye, after the rats were fasted overnight and anesthetized using ketamine. Blood was centrifuged at 3,000 rpm for 10 minutes, part of the plasma be separated, and performed tests: SOD activity (using the ELISA Kit Rats SOD, Sunlong Biotech), MDA (malondialdehyde Rats ELISA Kit, Sunlong Biotech), C-RP (Rats C-Reactive Protein ELISA kit, Sunlong Biotech), and IL-6 (interleukin 6 ELISA kit Rats, Sunlong Biotech).

2.5. Statistical Analysis

Obtained data were then analyzed by Analysis of Variance (ANOVA), and followed by Duncan test when there was significantly different at the level of 5%.

3. Results

After 2 weeks of intervention, the CREE significantly increase the activity of SOD of 111.46 to 137.60 ng / ml *(P = 0.003)* in atheroschlerosis rats plasma. Statin had no effect on SOD, but CREE plus statin was able to increase SOD from 111.78 to 150.95 ng/ml *(P=0,008),* compared to atherosclerotic rats that were given feed rats.

The plasma MDA concentration of atherosclerosis rats decreased from 3.44 to 2.38 nmol/l *(P = 0.031)* after the rats were given CREE for 2 weeks. Giving of statin for 2 weeks also reduce levels of MDA of 3.27 to 2,056 nmol / l *(P = 0.015)*. Decreasing on MDA levels from 2.25 to 1.55 nmol / l *(P = 0.024)* also occurred in the giving of CREE plus statin for 2 weeks.

In this study, CRP levels in atherosclerotic rats that expressed early at 52.77 ng/ml, but after being given CREE for 2 weeks, the levels decreased from 58.69 to 44.44 ng / ml or 0.0444 mg / L *(P = 0.049).*

Significantly decreased of IL-6 levels occurred on atherosclerotic rats given CREE 2 weeks from 111.96 to 83.33 ng / ml *(P = 0.002).* Giving of statin 2 weeks also lowered IL-6 plasma levels of atherosclerosis rats from 109.68 to 80.79 ng/ml *(P = 0.007).* The combination CREE plus statin given for 2 weeks also reduce of IL-6 levels, from 106.76 to 80.18 ng/ml *(P = 0.003)* (Table 1).

Table 1. The SOD activity and MDA, CRP and IL-6 levels of atheroschlerotic rats

	CREE			STATIN			CREE+STATIN			FEED RATS		
	0	1	2	0	1	2	0	1	2	0	1	2
SOD (ng/ml)	111.46^{a}	129.17^{a}	137.60^{b}	112.74^{a}	111.79^{a}	118.10^{a}	111.79^{a}	120.24^{a}	150.95^{b}	103,96^{a}	106,04^{a}	96,46^{a}
MDA (nmol/l)	3.44^{a}	2.97^{a}	2.38^{b}	3.27^{a}	2.60^{a}	2.06^{b}	2.25^{a}	1.73^{a}	1.55^{b}	2,01^{a}	1,89^{a}	2,05^{a}
CRP (ng/mL)	58.69^{a}	53.88^{a}	44.44^{b}	55.36^{a}	67.64^{a}	60.57^{a}	60.79^{a}	52.93^{a}	48.29^{a}	36,25^{a}	59,13^{a}	41,88^{a}
IL-6 (ng/mL)	111.96^{a}	99.73^{a}	83.33^{b}	109.68^{a}	98.54^{a}	80.80^{b}	106.76^{a}	85.56^{a}	80.18^{b}	77,69^{a}	94,35^{a}	79,17^{a}

Notes: N=7 rats; 0, 1, 2, intervention time in weeks; CREE, cardamom rhizome ethanolic extract; statin, simvastatin.

4. Discussions

4.1. The Effects of CREE on SOD activity

The levels of flavonoids in CREE was 324.51 mg / g (Winarsi et al., 2015), almost tripling levels in cardamom leaf extract (130 mg / g) (Winarsi et al., 2012). The CREE flavonoids may contribute to increasing the activity of

SOD. Flavonoids are hydrogen-donating antioxidant and free radical scavenger of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (McPhail et al., 2003). Flavonoids also be metal-chelating, which limits Fe-redox active form, thereby protecting cells against oxidative damage (Gong et al., 2010). The cellular antioxidant defense mechanisms will be weakened due to illness, for example in atherosclerosis conditions.

However, the group that received CREE flavonoids, SOD activity was higher than the control. The SOD removed the superoxide radicals by accelerate the dismutation reaction. Thus, exogenous antioxidants of flavonoids act directly reduce the formation of free radicals or interact with endogenous antioxidants to produce synergistic effect to minimize the number of radicals.

In addition, the hydroxyl group at the flavonoids in CREE donates hydrogen atoms to free radicals, so it becomes less reactive radicals. Reduced free radical would suppress the activity of macrophages, so the work of ingestion was inhibited. With the reduction of free radicals, the SOD inactivation process can be prevented. In other words, the activity of SOD increased by flavonoids of CREE.

4.2. The Effects of CREE on MDA levels

The increased activity of SOD in plasma relating to decrease the amount of free radicals, including MDA levels. Mechanism flavonoids of CREE reduce MDA levels was unclear. However, De-Xing et al. (2004) reported that the polyphenols in wine reduce the absorption of MDA which contribute to atherosclerosis. Because absorption is not perfect, so the flavonoid compounds reach the large intestine along with the products produced by the metabolism of the bacteria in the large intestine, and then excreted through feces. Hertzog and Tica (2012) adds that the flavonoids provide beneficial effects in the human body, which is reflected by the discovery of a number of flavonoids and monophenol in feces. The role of flavonoid CREE similar to wine polyphenols, which prevent the absorption of MDA, so that levels of MDA plasma low.

Decreased levels of MDA by Simvastatin proved that cholesterol-lowering drugs also indicates it works as an antioxidant against lipid peroxidation by reducing the formation of MDA. Several studies have reported that treatment with simvastatin may reduce oxidation of lipoproteins and repair the damage caused by free radicals (Ky et al., 2008; Mason et al., 2004). According to Moon et al. (2014) simvastatin therapy lowered levels of MDA and in parallel lowering cholesterol levels. However, Molcányiová et al. (2006) contradict it because in his research he uses only statin to lower cholesterol levels, but there was no reduction in the levels of MDA. MDA is three carbon di-aldehyde highly reactive, which is produced as a by product peroxidation of polyunsaturated fatty acids. Because it can be assumed that the decrease in MDA levels by simvastatin was a side effect of the reduction in lipid peroxidation.

The flavonoids in CREE may cooperate and work together to minimize the formation of free radicals, so that MDA levels decreased. Decreasing MDA levels occurred in rats given CREE was 31%, simvastatin was 37%, and a combination of CREE plus simvastatin was 31.2%.

4.3. The Effects of CREE on C-RP levels

C-reactive protein (CRP) is a chronic inflammatory biomarkers and risk factors are sensitive to cardiovascular disease (CVD). One of the acute-phase reactant is secreted by the liver in response to high levels of inflammatory cytokines such as IL-6 and IL-1β (Calabro et al., 2003). High CRP levels reflect poor immune status, associated with increased risk of CVD, therefore high levels of CRP can be used as a more sensitive predictor of acute cardiovascular events in comparison with other biomarkers, such as total-cholesterol and LDL- cholesterol (Verma et al., 2006).

Some researchers report that CRP concentration is inversely related to the intake of foods rich in polyphenolic antioxidants such as flavonoids (De Bacquer et al., 2006; Esmaillzadeh et al., 2006). In this study, CRP levels in rats decreased significantly, this may be the role of flavonoidsof CREE.

One of the mechanisms of dietary flavonoids to reduce levels of CRP through its antioxidant properties (Barnes et al., 2000). Some researchers believe that flavonoids are effective ROS scavenger and inhibits lipid peroxidation through chelation of transition metal ions (Srichairatanakool et al., 2006; Leopoldini et al., 2006) or can be as radical chain-breaking antioxidant (van Acker et al., 2000). Blake et al. (2003) and Ridker (2003) states that the cutoff CRP

levels over 3 mg/L can be used as an independent predictor of cardiovascular risk in the future. However, with the end of the study the rats CRP levels at 0.0444 mg/L, so that it can be concluded that the condition of this rats did not develop to CVD.

4.4. The Effects of CREE on IL-6 levels

Currently, inflammatory mediators implicated in the pathogenesis of atherosclerosis, including cytokines, chemokines, vasoactive molecules and growth factors. Significant decreased levels of IL-6 occurred on atherosclerotic rats given CREE. Similar findings by Xie et al. (2012) that the levels of IL-6 were significantly decreased in the peritoneal macrophages of rats were given blueberries contain polyphenols like anthocyanin and proanthocyanidin. The levels of IL-6 rats fed blueberries, lower than rats fed a standard feed. Anthocyanin and proanthocyanidin are bioactive compounds in blueberries responsible as a protection against vascular disease (Neto, 2007). However, according to Manach et al. (2005) polyphenol absorption is very low, so that the compound is not likely to be found in the body. Blueberries effectively inhibit production of inflammatory cytokines IL-6, through inhibition of NF-kB activation by reducing phosphorylation of p65 and NF-kB protein IKB, and inhibit phosphorylation of p38 MAPK and JNK.

As an antioxidant, flavonoids have a structure that is ideal as scavanger radicals that is the presence of more than one phenol compound, which is composed of an aromatic group and a hydroxyl group, and the presence of conjugated double bonds (Aulanni'am et al., 2012). In addition to acting as an antioxidant, flavonoids also acts as an immunomodulator through inflammatory response. Flavonoids modulate the inflammatory response by decreasing the activity of cyclooxygenase-2 (COX-2), lypoxygenase and induces the enzyme nitric oxyde synthase (iNOS). Other anti-inflammatory mechanism, flavonoids can inhibit the production of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1, IL-2, IL-6, IL-8, and IL-12. Inhibition of COX-2 and iNOS activation is achieved through the suppression of nuclear factor kappa B (NF-κB) (Jurenka, 2009). Flavonoids inhibit the production of cytokines such as IL-6 by lowering regulatory intercellular signaling proteins such as protein kinase C (PKC). Inhibition of PKC regulation occured because flavonoids can inhibit phosphorylation of phosphatidylserine, a phospholipid that was a compound that plays a role in the activation process through the PKC pathway diacylglycerol.

Flavonoids function as scavenger superoxide anion and hydroxyl radicals. Flavonoids donate a hydrogen atom to the peroxide radicals and radical form of flavonoids (which easily react with free radicals), which will meet with other flavonoid radical, to become stable molecules, thus lengthening chain radical reaction stop (Gusti, 2013). Using of flavonoid antioxidants can neutralize free radicals in cells, so there is no damage sustained.

Currently atherosclerosis is seen as an inflammatory disease of blood vessels, is characterized by the accumulation of lipids, and inflammatory cell infiltration (Libby, 2002). Recent clinical trials have demonstrated that CRP is a strong independent predictor of cardiovascular events. CRP is an acute phase reactant in humans, primarily derived from hepatocytes in response to IL-6 and then secreted into the systemic circulation. Therefore, decreasing the levels of IL-6 associated with lower CRP levels.

Giving of simvastatin for 2 weeks also lowered of IL-6 plasma levels. Simvastatin significantly lowered CRP-induced release of IL-6. Thus the decrease in levels of IL-6 was associated with decreasing levels of CRP by simvastatin. IL-6 is neuroprotective and neurotoxic. Decrease in CRP levels proves that simvastatin protects rats from the development of atherosclerosis in the direction of nerve damage.

IL-6 is a glycoprotein and is a pleiotropic cytokine with power, and a central mediator of the acute phase response of immune cells, with a wide range of due using simvastatin (Davignon and Mabile L. 2001). IL-6 can induce and inhibit growth, but it was also able to induce differentiation. IL-6 is produced by many types of cells such as monocytes, macrophage, fibroblasts, ceratinocytes, endothelial cells, mesangial cells, chondrocytes, osteoblasts, smooth muscle cells, T cells, B cells, granulocytes, mast cells and certain tumor cells (Suzuki *et al* ., 2009). The normal IL-6 value is 1 pg/ml (= 0.001 ng/ml), but it will increase in the obesity, physical activity, women menstruation, acute hyperglycemia, during and after surgery, as well as in patients with atherosclerosis (Fisman and Tenenbaum, 2010).

Statins inhibit IL-6 mediated inflammation through regulation of endogenous cholesterol synthesis and isoprenoid reduction (Omoigui, 2007). Polyphenol compounds found in plants (including cardamom) inhibits IL-6

directly inhibits signal transduction pathways. Bonetti et al. (2003) adds that the anti-inflammatory effects of statins are attributed to multifaceted mechanisms, including inhibition of cell cycle progression, induction of apoptosis, reduction of cyclooxygenase-2 activity and a biphasic, dose-dependent effect on angiogenesis.

The combination of CREE plus statin given for 2 weeks also significantly reduce levels of IL-6. Flavonoids of CREE synergy with simvastatin improve the immune system of atherosclerosis rats, with lower levels of IL-6 in plasma. Thus the decrease in levels of IL-6 atherosclerosis rats can occur with the use of CREE 25%, simvastatin 26%, and combinations of CREE and simvastatin 25%. Given the use of natural materials have fewer side effects, then to repair the immune system of patients with atherosclerosis CREE will be an option.

5. Conclusion

Cardamom rhizome ethanolic extract could improve antioxidant status by increasing SOD activity and lower levels of MDA plasma. Flavonoids were also able to improve the immune status of atherosclerosis rats by lowering levels of CRP and IL-6 plasma. The effect of Cardamom rhizome ethanolic extract on SOD and IL-6 was same with the effects of its combination with simvastatin, but to avoid any undesirable effects, it was advisable to use CREE. It is possible that in the future, the cardamom rhizomes would be formulated to become of functional drinks for patients with atherosclerosis.

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